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ADDITION OF SERUM TO ELECTROPORATED CELLS ENHANCES SURVIVAL AND TRANSFECTION EFFICIENCY

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SUMMARY: Optimal electroporation efficiency of many cell types is associated with poor survival. We show that serum rapidly reseals the membranes of electroporated cells and that timely addition of serum following electroporation can improve cell survival and transfection efficiency.

The utility of electroporation for incorporation of exogenous molecules into living cells depends upon the formation of a permeable state in the cell membrane. The degree of permeation depends upon the field strength and duration of the applied electric pulse; higher field strengths or longer pulse durations result in increased permeation (1). However, this increase is associated with increased cell death (2). Thus, conditions for maximizing the number of viable permeated or transfected cells are often tightly restricted and may be associated with considerable cell loss (2-4).

Electroporation conditions currently in use provide DNA transfection frequencies that are adequate for many studies, but improved efficiencies are needed for studies with rare cells such as hematopoietic stem cells, rare events such as homologous recombination, nonselectable genes, or all three (5). We found that rapid sealing of electroporated cells with serum could be used to advantage in improving survival and increasing the percentage of hematopoietic cells exhibiting transient expression of a β-galactosidase marker (pCMVβ) following electroporation.

MATERIAL AND METHODS

Cells and Medium: The murine myelomonocytic leukemia cell line WEHI-3B (D+) and the hematopoietic stem cell line FDP-A4 (7) were gifts of T.M. Dexter (Paterson Institute, Manchester, England). WEHI-3B cells were grown in Modified McCoys 5A medium with 10% fetal bovine serum (HyClone) at 37°C in 5% CO_2 in air. FDP-A4 cells were grown in Fishers medium with 20% horse serum and 10% WEHI conditioned medium (6). Serum used in the sealing experiments was the same as that used in the growth medium.

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Plasmid DNA: The mammalian expression vector, pCMVβ (8), was a gift of Grant MacGregor (Baylor College of Medicine, Houston). It was purified by CsCl gradient and used in supercoiled form as determined by agarose gel electrophoresis.

Electroporation: Exponentially growing cells were washed and resuspended in buffer (HeBS) consisting of 20 mM Hepes pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM dextrose. Cell density for electroporation was 3×10^7 cells/ml. DNA was added to a final concentration of 50 μg/ml.

Electroporations were performed with a BTX Transfector 300 (variable capacitance discharge) monitored with the BTX Optimizer. The optimizer measures resistance and records the peak voltage, the pulse duration (i.e), and a trace of the voltage decay curve for each discharge. Parallel plate electrodes (BTX P/N 470, gap = 2 mm) were covered with platinum foil and were mounted horizontally. The cell suspension was loaded between the plates; contact with the upper plate was maintained by surface tension. The electrodes were housed in plexiglas to prevent dangerous electrical shock. Capacitance settings were adjusted as necessary for different volumes of cells to yield a 7-8 ms pulse duration. All operations were performed at room temperature.

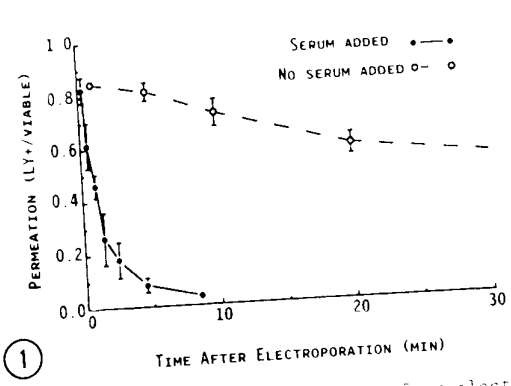
Permeation Assay: Permeation was measured using the lucifer yellow permeation assay of Presse *et al.* (9), modified such that dye was added after electroporation rather than being present during application of the electric pulse. An aliquot (10 μl) of electroporated cells was mixed with 1.1 μl of a 10 mM solution of lucifer yellow CH (Sigma) in HeBS and allowed to sit for 10 minutes. Since addition of serum increased the longevity of lucifer yellow permeated cells during subsequent handling and analysis and halted permeation (see Results), an equal volume of serum was added and the suspension was mixed and allowed to sit for an additional 5 to 10 minutes. The mixture was diluted with 1 ml of HeBS and the cells were pelleted and then analyzed by ultraviolet fluorescence microscopy. Results are expressed as the percentage of fluorescent cells among the total live cells in a given field containing at least 200 cells. Dead cells were very weakly positive, and were distinguishable by their diffuse outer membranes and were not counted.

Viability and Survival: Viability of electroporated cells was determined using eosin exclusion in a hemacytometer after 24 hours in complete medium under normal incubation conditions. Survival is expressed as the concentration of viable cells relative to control samples which were similarly treated and diluted but were not electroporated.

Transfection Efficiency: *LacZ* expression was measured 24 hours after electroporation by staining with the β-galactoside analog, 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), according to modified procedures of MacGregor *et al.* (10). Briefly, the cell suspension was pelleted, washed once with PBS, fixed with 0.2% formaldehyde, 0.4% glutaraldehyde in PBS for 5 minutes at 4°C, washed and pelleted in PBS, and stained in 100 μl of a mixture of 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM magnesium chloride and 1 mg/ml X-gal in PBS for 20 hours at room temperature. The percentage of plasmid expressing blue cells was determined using a hemacytometer.

RESULTS AND DISCUSSION

As shown in Figure 1 (upper curve), electroporated WEHI-3B cells kept in buffer at room temperature seal slowly with respect to lucifer yellow permeation. After 60 minutes, roughly half of the original permeated cells remained permeable. The slow decline in permeability allowed us to measure permeation by adding dye to a small portion of the cells after electroporation, thereby avoiding the need to add dye into the bulk of the cells during electroporation (see Materials and Methods). Sustained permeability of electroporated cells to various compounds and dyes has also been documented by others (11-13).



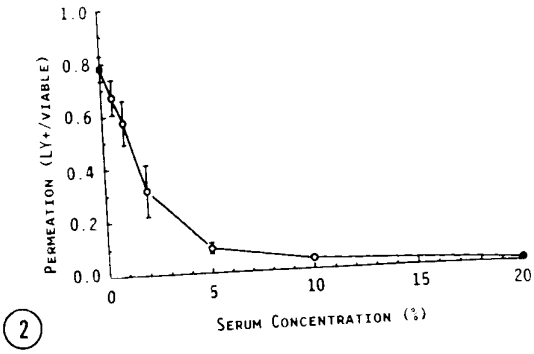


Figure 1. Immediately after electroporation (650 V/cm) serum was or was not added as indicated. Lucifer yellow was added for determining the permeability of cells at the indicated time points. Each point represents the mean and standard error for 3 experiments; absence of error bars indicates a standard error less than the symbol width.

Figure 2. Following electroporation at 650 V/cm, cells were mixed with serum or serum in HeBS to yield the final concentrations indicated. Ten minutes later lucifer yellow was added for determination of permeability. Each point represents the mean and standard error for three experiments.

When serum was added before lucifer yellow to electroporated cells, a marked reduction in permeation was observed. The rate of sealing was examined by adding lucifer yellow at measured intervals after adding serum to electroporated cells (Figure 1 - lower curve). Within 10 minutes at a serum concentration of 20%, cells were virtually impermeable to lucifer yellow. Parallel curves were obtained with FDP-A4 cells for both the sustained permeability and rapid sealing using horse serum (data not shown). The use of serum improved the permeation assay by allowing us to consistently stop the incorporation of dye after a chosen interval and by improving the vitality of permeated cells during analysis (see Materials and Methods).

The dose response relationship shown in Figure 2 indicates that a 20% serum concentration was fully effective for sealing electroporated WEHI-3B cells after 10 minutes, but even a 2% serum concentration had a significant effect on lucifer yellow permeation. As a preliminary step in characterizing the responsible factor(s), dialyzed serum was tested and found to be approximately 20% as effective as undialyzed serum, ie. dialyzed serum at a concentration of 10% gave a value for permeation similar to that obtained with a 2% concentration of undialyzed serum. Furthermore, after ultracentrifugation of whole serum (14 hours at 45,000 rpm) and separation into six equal fractions, all of the fractions showed sealing activity. The clear uppermost fraction and the dense bottom fraction were about 70% and 8 fold as effective, respectively, as whole serum, based upon comparison of appropriate dilutions. The activity of the uppermost fraction was only partially reduced by heating at 100°C for 5 minutes; the bottom fraction, diluted 1/5, gelled during this treatment. The loss of activity by dialysis suggests involvement of a low molecular weight component(s),

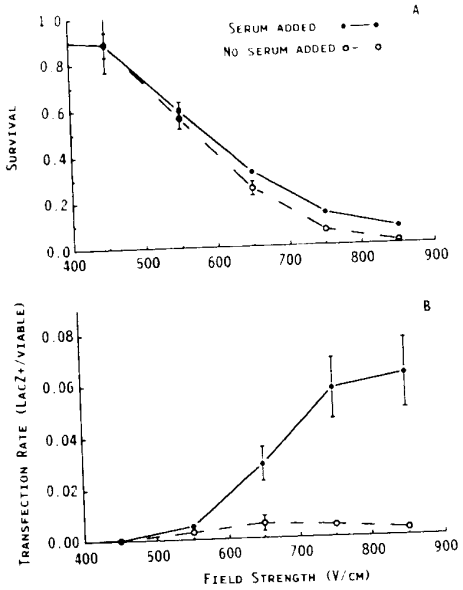


Figure 3. Survival (A) and transfection rate (B) were measured 24 hours after electroporation at various field strengths. See text for details. Each point represents the mean and standard error for 4 experiments

and the concentration of a factor(s) by ultracentrifugation suggests that a high molecular weight component(s) is also involved in the sealing effect of serum.

To analyze the effect of serum on survival and transfection efficiency, a series of electroporations were performed at increasing field strengths. After each electroporation, equal aliquots of electroporated cells were either mixed immediately with serum or allowed to sit in the electroporation medium at room temperature for ten minutes. Cells were then transferred to complete medium and incubated for 24 hours, at which time they were analyzed for survival and transfection efficiency. A modest but significant improvement in survival was found in serum treated samples at the three highest field strengths, 650, 750, and 850 V/cm (Figure 3A). The improvement in survival was accompanied by a more pronounced improvement in transfection efficiency among surviving cells (Figure 3B). Serum treatment provided an enriched population of transfected cells at the higher field strengths. These data imply that serum rescues a population of cells which are more likely to be transfected than the cells which survive without serum.

The net transfection efficiency (calculated as the product of the transfection efficiency of surviving cells times the survival rate) peaked at 550 V/cm for untreated cells and at 650 V/cm for serum treated cells. The latter peak was nearly 6 fold higher than the former (0.94% vs 0.16%).

Knutson and Yee (14) reported that electroporation of human carcinoma cells in complete medium (containing 10% FBS) gave equivalent DNA transfer to electroporation in PBS. When we added serum before electroporation at a field

strength of 700 V cm, we obtained similar results to those obtained when serum was added immediately after electroporation. The survival was 11 ± 1% vs 14 ± 1% (mean ± SE), and the transfection rate was 4.5 ± 0.7% vs 3.8 ± 1.4%, respectively. At this field strength with a ten minute postincubation in buffer, the survival was 6.4 ± 0.6% and the transfection rate was 0.5 ± 0.1%. These results show that serum does not significantly interfere with the transfer of DNA into the cells even when present during application of the electric pulse.

The sustained permeability seen with lucifer yellow after electroporation was not seen with DNA. When DNA was added immediately after electroporation in buffer, no transfectants were obtained (detection level 5 x 10^{-7} cells). Apparently DNA entry into the cell occurs within seconds of, if not during, the electric discharge. These findings are consistent with recent observations of Chang and Reese (15). Using electron microscopy of freeze fractured electroporated red blood cell membranes, they report that large pores (20-120 nm in diameter) develop within 40 ms and almost completely disappear within 10 seconds of the electric pulse. These short-lived large pores could be sites of entry for macromolecules such as DNA. The possible persistence of smaller pores (<3 nm) could not be determined within the resolution of their technique.

In the initial use of electroporation for DNA transfer, it was reported that a postincubation period in the electroporation medium containing DNA was necessary for DNA transfection, implying that during this period DNA uptake was occurring (16). Most DNA transfection protocols have subsequently incorporated a 10 minute incubation period for cells in buffer after electroporation (e.g. 2,17). However, Rabussay *et.al.* (18) showed no benefit of postincubation for DNA transfection of Cos7 cells, and several investigators follow protocols which do not employ the 10 minute postincubation period (14,19). Since there was no interference of DNA transfection with rapid sealing of membranes with serum, and since dilution of DNA upon transfer of cells to complete medium has been used effectively, the desirability of a 10 minute postincubation period for all cell types is not substantiated.

For DNA transfection there is as yet no clear mechanism of transmembrane passage. The effects of method modifications cannot be readily predicted, and improvements must come through empirical inquiry. Promising improvements have recently been reported with radio frequency electrical pulses (20), and with electroporation in hypo-osmolar medium (21). Preliminary tests indicate that serum may be combined with hypo-osmolar medium to yield further improvement for transfection of WEHI-3B cells (unpublished results).

In conclusion, serum contains components which rapidly reseal the membranes of electroporated cells to permeation by lucifer yellow. This effect may be used as a means to halt continued diffusion of small molecules into and presumably out of permeable cells, to improve survival of highly permeated cells, and somewhat unexpectedly, to increase the DNA electrotransfection efficiency in

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VERI-1B cells. Further investigation of the factors in serum which promote membrane sealing may help to shed light on the nature of the membrane permeability arising from electroporation.

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